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During the first year of work on this Proposal, we determined that the nCLU protein in human breast cancer cells is translated starting at an AUG-34 start site from within its mRNA. This start site meant that this protein is translated at an unique site from that of sCLU. Furthermore, we showed that nCLU mRNA is created by alternative splicing, exon II is omitted and exons I and II are spliced together. The loss of estrogen receptor in C4:2W cells correlated with a higher level of nCLU protein, and concomitant low levels of Ku70 when compared to the parental estrogen-dependent T47D breast cancer cell line.

Our previous data indicated that the N-terminal coiled-coil (Nterm) and C-terminal coiled-coil domains of nCLU interact with each other. Here, we demonstrated that conditional over-expression of the Nterm in MCF7 breast cancer cells results in radioresistance at clinically-relevant doses of IR (0.5 and 1 Gy). This finding, in combination with our data that nCLU is a pro-apoptotic protein, leads us to conclude that Nterm acts as a dominant-negative deletion mutant of nCLU. These data are consistent with our original hypothesis.

In conclusion, our data indicate that the nCLU protein plays an important role in breast cancer cell radiation survival, and that the efficiency of radiation therapy of breast cancer may be a function of the estrogen dependency of the particular cancer.

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## Table of Contents

Cover.....	
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	10
Reportable Outcomes.....	10
Conclusions.....	10
References.....	11
Appendices.....	12

## Introduction

Estrogen deprivation is the main component of many successful hormone-based therapies of breast cancer (6). Withdrawal from estrogen or treatment of breast cancer cells *in vitro* with estrogen antagonists, such as tamoxifen, results in significant increases in population doubling times (i.e., growth suppression) and high apoptotic indices (3, 7). In this project, we will concentrate on the apoptotic aspect of estrogen withdrawal. Previous studies in our lab demonstrated that MCF-7:WS8 breast cancer cells had increased levels of clusterin (CLU) protein forms, 24 – 48 h after incubation in estrogen-free RPMI medium. We also demonstrated that nuclear clusterin (nCLU) was a potential “death gene”(11). A number of apoptotic tissues and cell lines displayed elevation of CLU levels (1, 2). Some researchers argue that only surviving cells induce CLU synthesis (4, 5, 10). However, our experiments show that transfection of MCF-7:WS8 breast cancer cells with nCLU results in low survival (11). In contrast, transfection of MCF-7 cells with full-length clusterin (which will primarily produce secretory clusterin (sCLU) did not result in growth inhibition or loss of survival of transfectants (11). In this project, we propose a detailed series of investigations to examine whether cells that induce clusterin following estrogen withdrawal also undergo cell death and the effect of estrogen-independence on the steady-state level and apoptotic function of clusterin. We are also exploring the function of clusterin in estrogen deprivation-induced cell death using a proposed dominant-negative nCLU construct (ie., N-terminal portion of the CLU protein).

## Body

*Task 1.* To determine whether apoptosis occurs in cells that have elevated levels of nCLU after either estrogen deprivation or tamoxifen exposures (months 1-10).

- a. Perform estrogen withdrawal experiments with MCF7:WS8 cells and determine the levels of nCLU before and after estrogen withdrawal by Western blotting and immunocytochemistry (months 1-6).
- b. Perform simultaneous nCLU/TUNEL staining, and quantify the results using microscopy and FACS/flow cytometric analyses (months 3-10).
- c. Explore the effects of tamoxifen on nCLU induction/translocation and perform simultaneous nCLU/TUNEL staining in tamoxifen-treated cells (months 6-12).

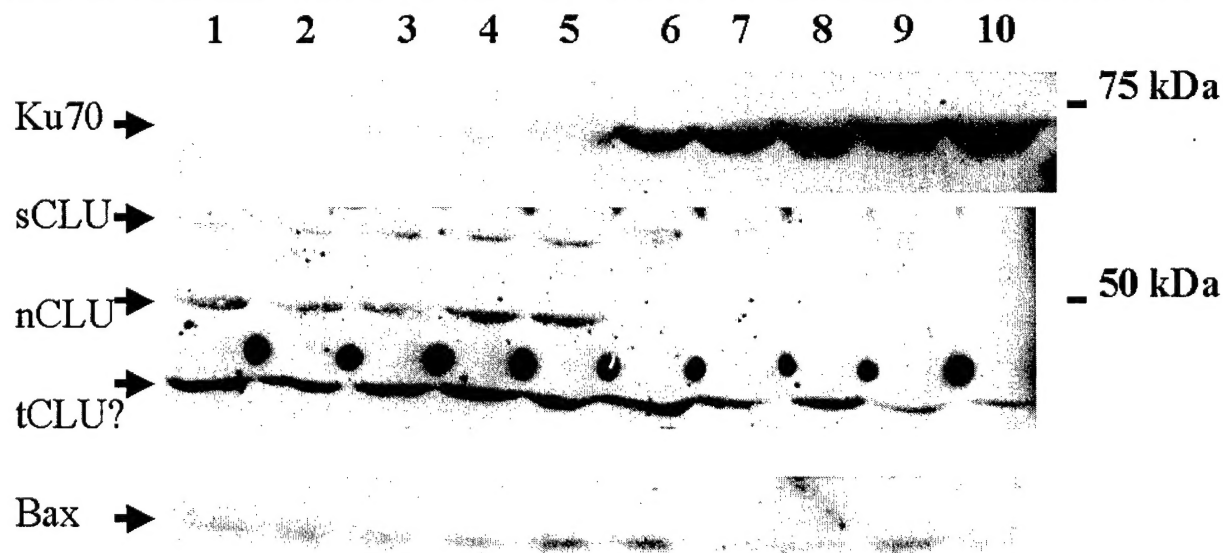
**Progress and problems encountered.** We performed estrogen withdrawal and tamoxifen treatment experiments on various estrogen-dependent and -independent breast cancer cell lines. Whole cell extracts from treated and untreated cells were analyzed for CLU expression by Western blotting using H-330 clusterin antibody (Santa Cruz). This polyclonal antibody detects a 49 kDa human nCLU and a 60 kDa sCLU forms. The representative results are shown on Figure 1. Estrogen-dependent T47D breast cancer cells line and its estrogen-independent C4:2W derivative clone were used (9). Surprisingly, T47D showed very low expression of nCLU as compared to C4:2W. Expression of sCLU was detected only in the presence of 1 nM estradiol without tamoxifen (Figure 1, lanes 6 and 9). In addition to nCLU and sCLU, the H-330 antibody detected an ~42 kDa protein that was different from the previously known 35-40 kDa glycosylated subunits of mature sCLU. The identification of this protein, and whether it is a nonspecific cross-reacting protein, is pending.

Another surprise came when we analyzed T47D and C4:2W extracts for Ku70 and Bax proteins. Ku70-nCLU protein-protein interaction was described previously (11-13). Interaction between Ku70 and Bax and its relevance to the regulation of apoptosis was described in Dr. S.

Matsuyama's (Medical College of Wisconsin) work (2002, manuscript in review). In T47D cells, low steady-state levels of the nCLU protein correlated with high levels of Ku70. In C4:2W cells, however, relatively high levels of nCLU were accompanied by very low levels of the Ku70 protein, a protein involved in DNA-PK-dependent nonhomologous end joining. Since Ku70 is an essential part of the DNA double strand break repair machinery, we expect C4:2W to be more radiation-sensitive compared to T47D. The significance of nCLU-Ku70 interaction remains to be determined, and we are analyzing these cells under these conditions to see if estrogen exposure increases radiation sensitivity. Our preliminary data on Figure 1 indicate that induction of nCLU may initiate Ku70 degradation. If this hypothesis is true, we expect an increase in Ku70 protein level in C4:2W when nCLU expression is inhibited by specific interfering RNAs (iRNAs). Alternatively, Ku70 down-regulation in C4:2W cells might be nCLU-independent or occur on the transcription or translation levels. A nCLU-containing adenovirus vector, described in Task 2, will allow us to determine the effect of nCLU over-expression on Ku70 protein levels. We expect decreases in Ku70 protein levels, and subsequent radiation sensitivity of T47D cells after nCLU transduction using the adenovirus vector. At 1 nM of estradiol, the overall growth rate of T47D cells is 1.5- to 2-fold higher than that of C4:2W (9). Similar differences were observed with Ku70 knockout versus wild-type cells, probably due to ~2-fold higher basal rate of apoptosis in the Ku70  $-/-$  cells (S. Matsuyama, 2002).

We also found an interesting correlation between the expression of Bax pro-apoptotic protein and estrogen in T47D cells. Contrary to the commonly accepted model that Bax is required for apoptosis, we detected down-regulation of Bax protein when cells were incubated either with 1  $\mu$ M of tamoxifen or in the absence of estrogen (Figure 1, lines 7, 8 and 10), as compared to T47D cells grown in the presence of 1 nM estradiol (Figure 1, lines 6 and 9). No changes in the steady-state level of Bax were detected in estrogen-independent C4:2W cell extracts. The physiological significance of Bax down-regulation by estrogen is not clear. We are currently determining the dependency of Bax protein levels on the dose of tamoxifen, as well as tamoxifen survival assay on the T47D and C4:2W cells.

With several forms of CLU detected by H-330 antibody and insufficient amount of polyclonal anti-nCLU antibody described in (11) due to rabbit's death, the proposed immunocytochemical staining of cells specifically for nCLU, as proposed in Task 1, was not possible. Therefore, we propose to generate mouse monoclonal antibody to the N-terminal part of nCLU. To do this, we needed to determine the start site of nCLU and tCLU forms (Figure 1).

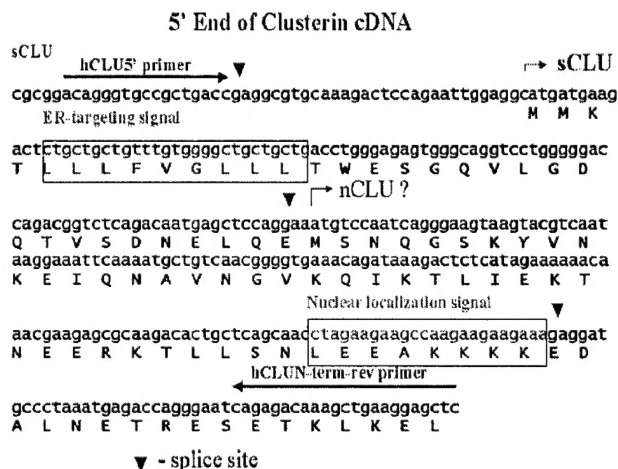


**Figure 1. Estrogen dependency correlates with specific patterns of the expression of CLU, Ku70 and Bax proteins.** Western blot analysis of whole cell extracts from breast cancer cell

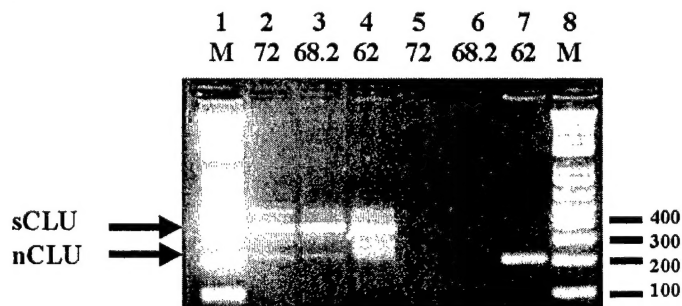
lines. Lanes 1-5, T47D:C4:2W ER-negative clone; 6-10, T47D parental ER-positive cell line. Lanes 1 and 6, cells incubated with whole 5% fetal bovine serum (FBS); 2 and 7, estrogen-free FBS with 1  $\mu$ M tamoxifen; 3 and 8, same as 2 and 7 + 1 nM estradiol; 4 and 9, 1 nM estradiol; 5 and 10, estrogen-free FBS. Note the down-regulation of Ku70 in ER-negative T47D:C4:2W cells, sCLU and nCLU in ER-positive T47D cells and low steady-state levels of Bax in T47D cells in the absence of estradiol or in the presence of tamoxifen.

In order to determine how estrogen regulates nCLU *versus* sCLU, we first needed to elucidate the mechanism of nCLU synthesis. When we examined the exon/intron structure of the CLU gene, we found that the first AUG of sCLU and the potential start site of nCLU of human *clu* gene are located in exon II and exon III, respectively (Figure 2A). To determine whether exon II could be spliced out and exons I and III joined together, we performed an RT-PCR using primers to exons I and exon III. The results are shown on Figure 2B. All visible RT-PCR products were isolated from the agarose gel and sequenced. One of them, a 220 bp fragment was, as we had hypothesized, the 5' end of the proposed nCLU-specific cDNA (Figure 2C). Subsequently, the full-length nCLU cDNA was isolated using nCLU-specific primer that spans exon I/exon III junction. This nCLU cDNA was translated in vitro (Figure 3, lane 8) resulting in a 49 kDa protein product as observed on the Western blot analysis of cell extracts. Based on these data, we concluded that nCLU mRNA is a result of alternative splicing and nCLU protein translation starts at AUG-34 (when AUG-1 is the first codon of sCLU). This work was presented on the 49<sup>th</sup> Radiation Research Society Meeting (Reno, NV, April 20-24) and I received the prestigious Marie Curie Award, given to a post-doctoral fellow based on scientific merit.

A

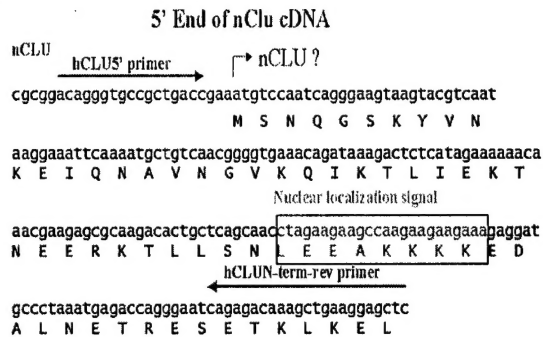


B





C



**Figure 2. nCLU is produced by alternative splicing.**

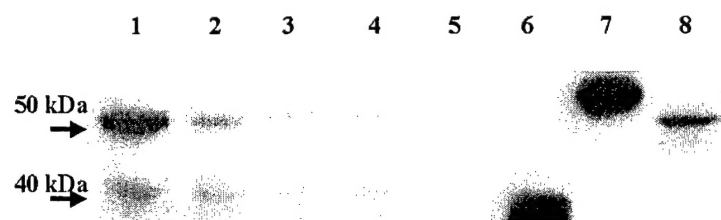
**A. Structure of the 5' end of sCLU cDNA.** Splice sites are indicated as black triangles. Small arrows show translation start sites of sCLU and nCLU. Endoplasmic reticulum targeting leader peptide (ER-targeting signal) and nuclear location signal are shown in boxes. Long arrows indicate primers used to amplify various splicing variants of CLU cDNA.

**B. 5' end of CLU mRNA undergoes alternative splicing.** 2% agarose gel showing CLU cDNA products of the RT-PCR using hCLU5' and primers (lanes 2-4). Total RNA from MCF7:WS8 breast cancer cells was used in the reverse transcription step with oligo(dT). The annealing of PCR stage was performed at 62, 68.2 and 72 °C as shown. Note a major 345 bp sCLU and a 220 bp nCLU products. Products longer than 345 bp are the result of insertion of an additional untranslated exons between exon I and exon II. All visible RT-PCR products were isolated and sequenced. Lanes 5-7 show nCLU-specific RT-PCR product amplified using hCLUN-term-rev primer and nCLU-specific primer that spans exon I/exon III junction and end of nCLU cDNA. This primer is currently used to design a quantitative RT-PCR method to detect the steady-state levels of nCLU mRNA.

**C. Sequence of the 5' end of nCLU cDNA that is the result of an alternative splicing event when exon II is omitted.**

To determine the potential mechanism for tCLU synthesis, we performed an in vitro translation of human sCLU mRNA (Figure 3) in the rabbit reticulocyte extract (Promega, Inc.). In addition to a 52 kDa sCLU protein precursor, we detected a 42 kDa protein, similar to the tCLU detected with H-330 antibody (Figure 3, lane 1). To distinguish between cap-dependent and cap-independent translation initiation of tCLU, we performed translation in the presence of 0.1 or 0.8 mM of m7GpppG cap analog (Figure 3, lanes 2 and 3). As we would expect in the case of cap-independent translation initiation, the sCLU/tCLU ratio dropped from 3.4 without cap analog to 2.6 and 1.2 at 0.1 or 0.8 mM of m7GpppG, respectively, as determined by Phosphoimager. No 42 kDa protein was detected in the absence of template (Figure 3, lane 5). Only 52 kDa protein was produced when methionines 115 and 116 were simultaneously mutated to isoleucine and glutamine, respectively (lane 7). The mRNA with ORF starting at methionine 115 produced an identical 42 kDa product (lane 6). No 42 kDa tCLU was produced when nCLU mRNA was translated (lane 8). These data indicate that tCLU protein is translated on the sCLU mRNA via cap-independent, possibly, internal ribosome entry site (IRES) mechanism. The tCLU ORF starts at AUG115 or AUG116. We do not have any evidence regarding the function of tCLU and its relevance to the cell growth regulation by estrogen in breast cancer cells. Therefore, further study of tCLU will not be a part of this proposal. Based on these data, we

propose using a peptide corresponding to the nCLU region between methionine 34 and methionine 115 as an antigen to generate nCLU-specific monoclonal antibody (see Conclusions).



**Figure 3. sCLU mRNA contains a potential internal ribosome entry site (IRES).** *In vitro* transcription-translation reaction in rabbit reticulocyte extract (Promega, Inc.) using following human CLU mRNAs as templates. Lines 1-4, – full-length sCLU mRNA; 5, – no template, 6, – CLU mRNA starting at Met115; 7, – sCLU with a MM115,116IQ mutations; 8, – CLU mRNA starting at Met 34. In lanes 1-3 sCLU mRNA is capped; lane 4 – no cap. Lanes 2 and 3, – translation in the presence of cap analog with following concentrations: lane 2 – 0.1 mM, and lane 3 – 0.8 mM.

**Task 2.** Determine the ability of the N-terminal coiled-coil domain of clusterin to inhibit apoptosis caused by either estrogen deprivation or tamoxifen exposure.

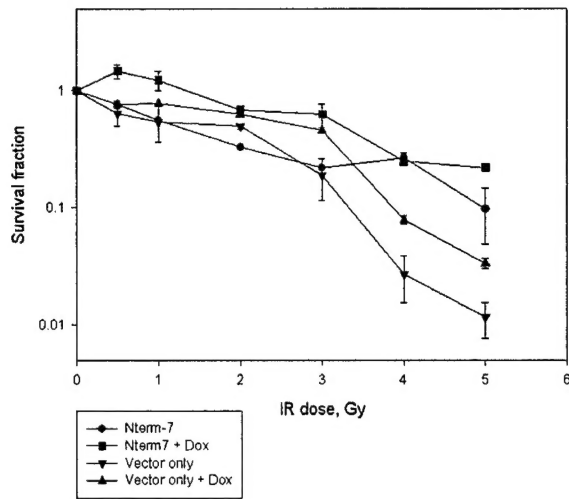
- a. Make a series of adenoviral vectors containing HA or HA-fused full-length clusterin, nCLU, N-terminal coiled-coil domain, central no-coil region, C-terminal coiled-coil domain and C-terminal no-coil region proteins (months 13-22).

**Progress:** We have made an adenoviral vector containing human nCLU cDNA with and without a mutation in the C-terminal coiled-coil domain. We have demonstrated by yeast two-hybrid assay that this mutation (Leu343 → Pro, L343P) abrogates nCLU binding to Ku70. We hypothesize that L343P mutation may also abrogate apoptotic/cytostatic functions of nCLU. We are currently at this step studying the effect of estrogen deprivation and tamoxifen treatment on the MCF7:WS8 breast cancer cells transduced with the adenovirus containing either wild-type or mutant nCLU.

- b. Transfect MCF7:WS8 cells with HA-clusterin fragments in the adenoviral vectors (months 23-36).
- c. Perform TUNEL and other cytological analyses, such as trypan blue exclusion, to determine apoptosis-related DNA fragmentation and the viability of transfectants (months 23-36).
- d. Select stably transfected clones from “c” above and determine their responses to estrogen deprivation or tamoxifen exposures (months 23-36).

**Progress (b-d):** In addition to the adenovirus vectors, we have made tetracycline (tet) – inducible constructs based on pTRE2 vector (Clontech). These plasmids contain human nCLU cDNA and the N-terminal part of human nCLU (Nterm). Our previous data show that this Nterm fragment can bind endogenous nCLU and, according to our hypothesis, act as a dominant negative mutant by protecting cells from nCLU-induced apoptosis. Stable clones of MCF7:WS8 cells expressing tet-inducible Nterm were established. Preliminary data demonstrate that the expression of Nterm results in 2-2.5 times increased resistance to clinically-relevant doses (0.5-1 Gy) of ionizing radiation (Figure 1). We are currently investigating whether Nterm can protect MCF7:WS8 cells against estrogen deprivation/tamoxifen-induced apoptotic and cytostatic effects.





**Figure 1.** Doxycycline-inducible expression of Nterm fragment of human nCLU (squares) in MCF7:WS8 cells results in 2-2.5 fold radioresistance at 0.5-1 Gy as compared to non-induced Nterm (circles) and vector-only transfectants (triangles). Colony forming assay was performed in triplicates. Bars represent standard error for each point.

*Task 3.* Determine differences in oral DMBA-induced mammary gland tumorigenesis between clusterin knockout mice (-/-), compared to either heterozygous (+/-) or wild-type (+/+) animals.

**Progress:** We are currently genotyping CLU knockout mice for the experiment. We have been experiencing problems with breeding of sufficient number of the CLU knockout females. Because of that, the number of animals used in the experiment may be scaled down.

- a. Genotype sufficient numbers of female mice from heterozygous breeding of the clusterin knockout colony. Approximately 210 animals, about 70 mice from each genotype will be used for the experiment (months 1-3).
- b. Administer DMBA orally to the clusterin -/-, +/- and +/+ mice, 50 animals of each genotype. Analyze the mice for the presence of mammary tumors 7-8 months later (months 4-15).
- c. Administer tamoxifen to half of the mice with tumors. Administer tamoxifen to the animals untreated with DMBA (20 mice of each genotype) (months 11-15).
- d. Analyze mice for the presence of mammary gland tumors (months 16-17).
- e. Perform histological analysis of DMBA-induced tumors in clusterin +/+, +/- and -/- animals (months 16-36)
- f. Genotype sufficient numbers of female mice (approximately 210 animals) as in "a" above for replicate experiments (months 8-10).
- g. Repeat DMBA and tamoxifen administrations (steps b – e from above) and examine mice for tumors (months 11-22).
- h. Genotype another 210 mice and repeat steps b – e. (months 20-36).

## Key Research Accomplishments

During the first year of the work on the Proposal, we have made the following accomplishments:

1. Identified the mechanism on nCLU biosynthesis by alternative splicing.
2. Demonstrated differential nCLU expression in estrogen-dependent vs -independent breast cancer cells.
3. Demonstrated the presence of internal translation initiation in the CLU mRNA.
4. Demonstrated that the conditional expression of the N-terminal coiled-coil domain of nCLU results in higher radioresistance of MCF7:WS8 breast cancer cells.
5. Generated adenovirus vectors containing wild-type and mutant nCLU.

## Reportable outcomes.

During the first year of work on the Project the following abstracts were presented:

**Konstantin S. Leskov, Chin R. Yang, Jing Li, Dmitri Klovov, Timothy J. Kinsella and David A. Boothman.** Clusterin revealed: alternative splicing results in pro- and anti-apoptotic ionizing radiation inducible forms of CLU. 49<sup>th</sup> Meeting of the Radiation Research Society, April 20-24, 2002.

**Konstantin S. Leskov, Jing Li, Dmitri V. Klovov, David A. Boothman** Synthesis of pro-apoptotic forms of Clusterin, an ionizing radiation-induced protein. Low Dose Radiation Research Workshop, Rockville, MD 2002.

The following manuscripts were submitted (currently in review):

**Sun, W., Sawada, M., Hayes, P., Leskov, K., Boothman, DA., and Matsuyama, S.** Ku70 suppresses the apoptotic translocation of Bax to mitochondria. 2002; Submitted to Mol. Cell Biol.

**Konstantin S. Leskov, Dmitry Y. Klovov, Jing Li, Timothy J. Kinsella and David A. Boothman.** Characterization of biosynthesis and structure of nuclear clusterin (nCLU). 2002; Submitted to J. of Biological Chemistry.

## Conclusions

Based on our data, we conclude that nCLU protein in human breast cancer cells is translated starting at AUG-34 from the mRNA that is separate from that of sCLU. This nCLU mRNA is created by alternative splicing when exon II is omitted and exons I and II are spliced together. The loss of estrogen receptor correlates with higher level on nCLU and low level of Ku70 proteins.

Our previous data indicated that N-terminal coiled-coil (Nterm) and C-terminal coiled-coil domains of nCLU interact with each other. Here, we demonstrated that conditional over-expression of Nterm in MCF7 cells results in radioresistance at clinically-relevant doses of IR (0.5 and 1 Gy). This finding, in combination with our data that nCLU is a pro-apoptotic protein, leads us to conclusion that Nterm acts as a dominant-negative deletion mutant of nCLU.

Due to the problems with the specificity of the available nCLU antibodies and new data obtained during the work on Task 1, we propose some changes in the Statement of Work for the following two years. These changes are to be made in Task 1, whereas Tasks 2 and 3 remain the same as stated in the original Proposal. In particular, we are expanding our model system from

the initially proposed MCF7:WS8 cells to a wider variety of estrogen-dependent and -independent breast cancer cell lines, including T47D and C4:2W. This will allow us to better explore the effects of estrogen and tamoxifen on the nCLU function in breast cancers.

Our working hypothesis is:

**We hypothesize that the induction of nuclear clusterin (nCLU) in breast cancer cells results in higher apoptosis rate. We also hypothesize that the loss of estrogen receptor results in higher steady-state level of nCLU, and it causes alterations in Ku70 and Bax protein expression. We also anticipate higher mammary carcinogenesis in clusterin knockout mice following DMBA treatment compared to wild-type or heterozygous animals, since potentially cancerous cells with heavily damaged genome are not eliminated by nCLU-dependent apoptosis in the CLU knockout animals.**

Based on this hypothesis, we propose the following Task 1.

- a. Generate mammalian expression vectors containing human estrogen receptor (ER), ER interfering RNA (iRNA)-expressing system and nCLU iRNA-expressing system as described in (8) (months 13-14).
- b. Perform transfection of C4:2W cells with ER and nCLU iRNA plasmids, transfect T47D cells with ER iRNA plasmid. Select stable clones and confirm the expression of ER protein in C4:2W cells and down-regulation of nCLU protein in C4:2W ER protein in T47D. (months 14-22).
- c. Determine cell growth response to estrogen and tamoxifen in the stably-transfected clones compared to vector alone transfectants. Determine the levels of Ku70 and Bax proteins and their intra-cellular distribution by Western blotting, and radiation sensitivity by clonogenic survival assay. (months 22-36).
- d. Generate mouse monoclonal antibody toward the N-terminal part of nCLU (amino acids 34-114) (months 13-24).
- e. Use anti-nCLU monoclonal antibody (depending on their quality and availability) to detect endogenous nCLU in T47D, C4:2W and MCF7:WS8 breast cancer cells following estrogen and tamoxifen treatments (months 24-36).

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## Appendix.

The following abstracts were supported by the DOD grant DAMD-17-01-1-0196.

**Konstantin S. Leskov, Chin R. Yang, Jing Li, Dmitri Klovov, Timothy J. Kinsella and David A. Boothman** Clusterin revealed: alternative splicing results in pro- and anti-apoptotic ionizing radiation inducible forms of CLU. 49<sup>th</sup> Meeting of the Radiation Research Society, April 20-24, 2002. \*

Clusterin (CLU) is an ionizing radiation (IR)-induced protein that is reported to have both pro- and anti-apoptotic functions. The major form of CLU protein is secretable (sCLU) and is induced by low doses of IR (2 cGy). Several groups have shown that sCLU acts as a protector against apoptosis. In addition, the existence of another, nuclear CLU form (nCLU) was proposed. We demonstrated that nCLU induced caspase-3-independent cell death upon over-expression in MCF7:WS8 and SV-40-immortalized mouse embryonic fibroblasts. We also demonstrated that the C-terminal coiled-coil domain of nCLU was responsible for cell death induction. Our data also indicated that the N-terminal coiled-coil domain of nCLU bound to the C-terminal coiled-coil domain and could promote either intra-molecular folding or oligomerization. We showed that endogenous nCLU protein was induced in the nuclei of IR-treated cells by higher doses of IR ( $\geq 1$  Gy). We hypothesize that nCLU protein induction may be necessary for the elimination of severely damaged cells. In this work, we have determined the mechanisms for nCLU synthesis. We isolated a cDNA for nCLU from IR-treated MCF7:WS8 cells. This nCLU mRNA was produced by splicing together exons I and III, and splicing out exon II that contained the first AUG and the endoplasmic reticulum-targeting signal of sCLU. As a result of alternative splicing, the AUG that was previously proposed to be a translation start site for nCLU protein became the first available start codon. We are currently investigating the expression of nCLU in mammalian cells, their functions regarding apoptosis, and their responses to IR, including regulation of splicing and translation initiation. We propose that CLU RNA message undergoes alternative splicing and internal translation initiation resulting in two pro- and one anti-apoptotic form of the protein. *This work was supported by DOD grant #DAMD-17-01-1-0196 to K.S.L.*

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**Konstantin S. Leskov, Jing Li, Dmitri V. Klovov, David A. Boothman** Synthesis of pro-apoptotic forms of Clusterin, an ionizing radiation-induced protein. Low Dose Radiation Research Workshop, Rockville, MD 2002.

Clusterin (CLU) is an ionizing radiation (IR)-induced protein that is reported to have both pro- and anti-apoptotic functions. The major form of the CLU protein is secreted (sCLU), and is induced by low doses of IR ( $\geq 2$  cGy). Several groups have shown that sCLU acts as a protector against apoptosis.

However, the existence of another, nuclear form of CLU (nCLU) was proposed and reported by us (Yang et. al., PNAS, 2000). We demonstrated that nCLU induced caspase-3-independent cell death upon forced over-expression in MCF7:WS8 and SV-40-immortalized mouse embryonic fibroblasts. We also demonstrated that the C-terminal coiled-coil domain of nCLU was responsible for cell death. Our recent data indicate that the N-terminal coiled-coil domain of nCLU bound to the C-terminal coiled-coil domain, promoting either intra-molecular folding or oligomerization. We showed that endogenous nCLU protein was induced in the nuclei of IR-treated cells by higher doses of IR ( $\geq 1$  Gy), and we hypothesized that nCLU protein induction may be necessary for the elimination of severely damaged cells. We recently determined the mechanisms for nCLU synthesis. We isolated a cDNA for nCLU from IR-treated MCF7:WS8 cells. This nCLU mRNA was produced by splicing together exons I and III, and eliminating exon II, which contained the first AUG codon and the endoplasmic reticulum (ER)-targeting signal of sCLU. In the result of this alternative splicing, translation started from the second in-frame AUG codon positioned in exon III leading to the production of the nCLU protein. We are currently investigating the expression of nCLU in mammalian cells, its functions regarding apoptosis, and its responses to IR, including regulation of splicing and translation initiation. We propose that CLU RNA message undergoes alternative splicing resulting in pro- and anti-apoptotic form of the protein. *This work was supported by DOE grant #DE-FG02-99EQ62724 to D.A.B. and by DOD grant #DAMD-17-01-1-0196 to K.S.L.*